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A multilocus phylogeny of the *Metarhizium anisopliae* lineage

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Abstract: *Metarhizium anisopliae*, the type species of the anamorph entomopathogenic genus *Metarhizium*, is currently composed of four varieties, including the type variety, and had been demonstrated to be closely related to *M. taii*, *M. pingshaense* and *M. guizhouense*. In this study we evaluate phylogenetic relationships within the *M. anisopliae* complex, identify monophyletic lineages and clarify the species taxonomy. To this end we have employed a multigene phylogenetic approach using near-complete sequences from nuclear encoded EF-1α, RPB1, RPB2 and β-tubulin gene regions and evaluated the morphology of these taxa, including ex-type isolates whenever possible. The phylogenetic and in some cases morphological evidence supports the monophyly of nine terminal taxa in the *M. anisopliae* complex that we recognize as species. We propose to recognize at species rank *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acridum* stat. nov., *M. lepidiotae* stat. nov. and *M. majus* stat. nov. In addition we describe the new species *M. globosum* and *M. robertsii*, resurrect the name *M. brunneum* and show that *M. taii* is a later synonym of *M. guizhouense*.

Key words: biocontrol, Clavicipitaceae, *Cordyceps*, entomopathogen, green muscardine, *Metacordyceps*

INTRODUCTION

The genus *Metarhizium* Sorokin is composed of anamorph entomopathogenic fungi that generally are greenish when conidiating on the corpses of their arthropod hosts or in axenic culture. They frequently are isolated from soils, parasitize a broad range of insect species representing numerous orders and are found throughout the tropics and temperate regions. Species from this genus are used as biological control agents to manage and prevent infestations of various species of superfamily Acridoidea, including locusts and grasshoppers (Lomer et al 1997, Milner 1997, Milner and Pereire 2000, Hunter et al 2001, Lomer et al 2001). In addition *Metarhizium anisopliae* (Metschn.) Sorokin, the type species of the genus, has been shown to be effective in the control of malaria-vectoring mosquitoes (Culicidae, Diptera). Two recent studies have estimated that applications of *M. anisopliae* could reduce the intensity of malaria transmission by 75% (Scholte et al 2005, Kanzok and Jacobs-Lorena 2006).

Liang et al (1991) were the first to confirm the connection of *Metarhizium*, long considered to be asexual, to the teleomorph genus *Cordyceps* (Fr.) Link (Clavicipitaceae, Hypocreales). They described *C. taii* Z.Q. Liang & A.Y. Liu and linked it developmentally to their newly described anamorph species, *M. taii* Z.Q. Liang & A.Y. Liu. This genus-level anamorph-teleomorph connection was substantiated further when Liu et al (2001) described a *Metarhizium* anamorph for *C. brittlebankisoides* Zuo Y. Liu, Z.Q. Liang, Whalley, Y.J. Yao & A.Y. Liu. This link has been supported by subsequent molecular phylogenetic studies (Liu et al 2002, Huang et al 2005a). The sexual states of *Metarhizium* species have been transferred from *Cordyceps* to *Metacordyceps* Sung et al (2007).

Before Tulloch's (1976) revision of *Metarhizium* 13 species and two varieties had been proposed (see www.IndexFungorum.org). Tulloch rejected three species described by Sorokin (1883) as defined according to the morphology of the hyphal bodies formed within the hemocoel of their respective insect hosts. Based on the illustrations accompanying the descriptions of *Metarhizium* spp. by earlier authors, Tulloch determined that some species were not members of the genus and she concurred with the synonymies proposed by others (Gams and Rozsypal 1973, Latch 1965, Speare 1912, Veen 1968). In the end Tulloch (1976) rejected most of the published names and reduced the genus to two species (*M. anisopliae* and *M. flavoviride* W. Gams & Rozsypal) including the type and one other variety (*M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* [J.R. Johnst.] M.C. Tulloch).

In the most comprehensive morphological and molecular phylogenetic treatment of *Metarhizium* to date Driver et al (2000) followed Tulloch's (1976) lead in recognizing species complexes for *M.*

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anisopliae and *M. flavoviride* in addition to *M. album* Petch, which was supported by Rombach et al (1987). However they were unable to assess the phylogenetic affinities of several taxa associated with *Metarhizium* that had been described since Tulloch's (1976) treatment. The taxa not studied by Driver et al (2000) included *M. pingshaense* Q.T. Chen & H.L. Guo, *M. guizhousense* Q.T. Chen & H.L. Guo and *M. taii* (anamorph of *Metacordyceps taii*). These taxa were described from China and have not been available for study until recently.

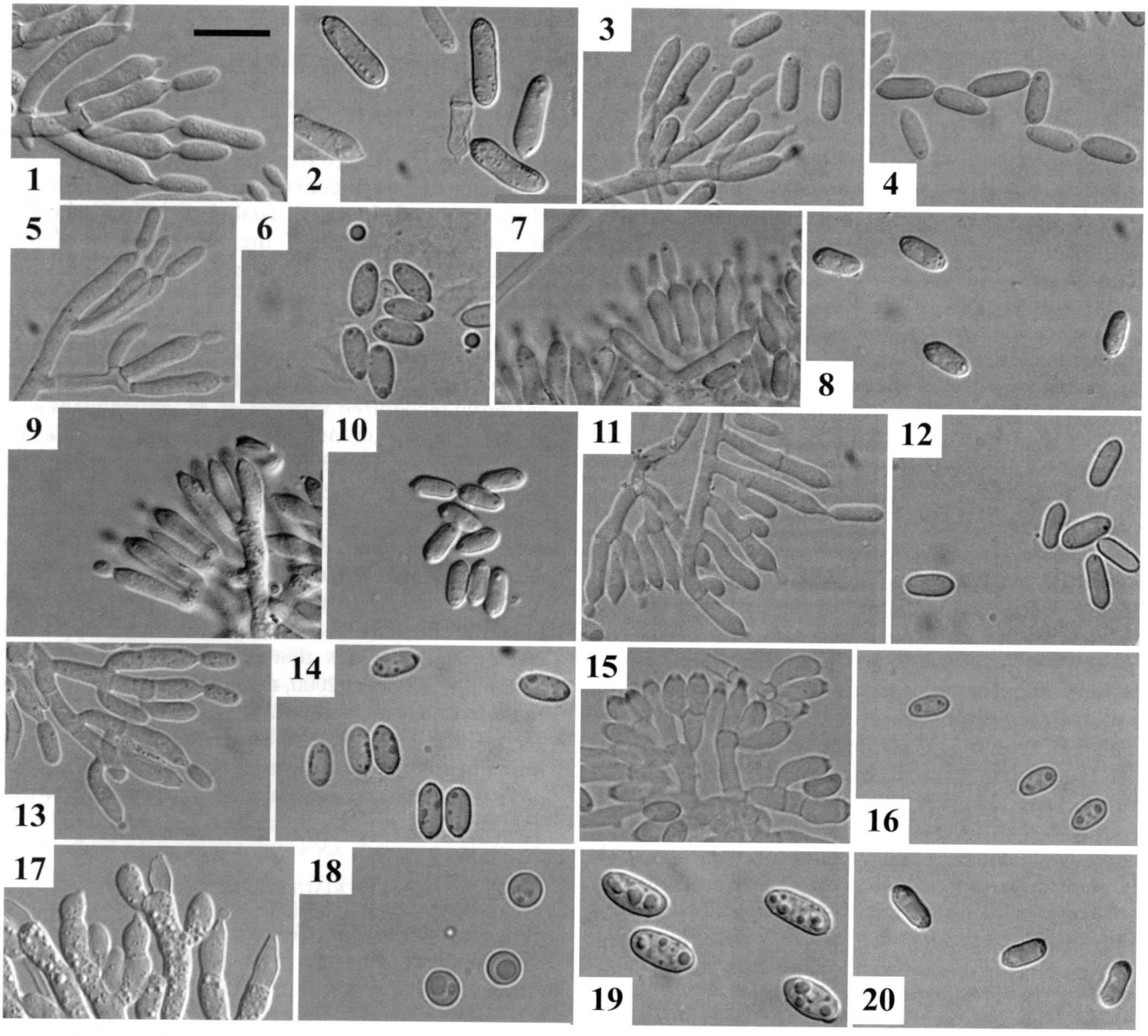
Driver et al (2000) expanded the circumscriptions of *Metarhizium anisopliae* and *M. flavoviride*. Applying the results of a phylogenetic analysis of the nuclear ribosomal internal transcribed spacer (ITS), Driver et al (2000) recognized eight lineages as varieties and one undetermined species group (i.e. *M. flavoviride* "Type E"). They identified and described varieties for both species complexes including four varieties of *Metarhizium anisopliae*, namely *M. anisopliae* var. *acridum* Driver & Milner, *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *lepidiotae* Driver & Milner (as *M. anisopliae* var. *lepidiotum*) and *M. anisopliae* var. *majus*. Driver et al (2000) restricted their descriptions of new lineages to varieties due to the limited resolution and support provided by the ITS sequence analysis. In their ITS phylogeny (Driver et al 2000 FIG. 1) all the terminal lineages that defined the varieties of *M. flavoviride* form an unresolved polytomy. Furthermore, the internal nodes within the clade containing *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* were unresolved as well. Isolates associated with the invalidly described *nomen nudum* "*M. anisopliae* var. *frigidum*" introduced by Rath et al (1995) also were included in Driver et al (2000). The ITS phylogeny of Driver et al (2000) showed an affinity between "*M. anisopliae* var. *frigidum*" and *M. flavoviride* var. *flavoviride*. However ITS sequence data did not provide significant support for their reciprocal monophyly despite morphological differences that support their distinction, hence Driver et al (2000) considered them synonymous. In a study that used a multigene phylogenetic approach Bischoff et al (2006) unambiguously resolved the three varieties of *M. flavoviride* and described *M. frigidum* J.F. Bisch. & S.A. Rehner (= "*M. anisopliae* var. *frigidum*") as a morphologically and phylogenetically well supported lineage within the *M. flavoviride* complex.

Huang et al (2005a, b) addressed the taxonomy and relationships of several taxa from China that were not included by Driver et al (2000). In these papers *Metarhizium guizhouense*, *M. pingshaense* and *M. taii* were synonymized with *M. anisopliae*. However, as in Driver et al (2000), Huang et al (2005a, b) derived their phylogenetic inferences exclusively from ITS, which performs poorly in resolving lineages within *Metarhizium*, especially within the *M. anisopliae* complex. The results reported by Driver et al (2000) and Huang et al (2005a, b) suggest that ITS sequences by themselves are of limited use for resolving the phylogenetic history of *Metarhizium*. However the phylogenetic analysis used in Bischoff et al (2006) demonstrated the effectiveness of this multigene approach and provides a more sensitive and robust means to identify *Metarhizium* lineages.

In this study we address and clarify the taxonomy and phylogenetics of the *Metarhizium anisopliae* complex that we consider to include all of the four currently recognized varieties of *M. anisopliae* as well as *M. guizhouense*, *M. pingshaense* and *M. taii*. To accomplish this objective we have taken a multigene phylogenetic approach to determine whether these previously recognized species and varieties are phylogenetically distinct. Furthermore we examined the morphology of isolates from all inferred lineages within the complex, using ex-type isolates whenever possible. In the cases of several well supported intrageneric lineages that are not morphologically distinct (Driver et al 2000, this study) we have adopted the genealogical concordance phylogenetic species recognition criterion (GC-PSR, Taylor et al 2000) as implemented by Dettman et al (2003) and O'Donnell et al (2000) to recognize species lineages within this complex.

MATERIALS AND METHODS

Fungal isolates.—The 57 isolates used in this study (TABLE I) were obtained from ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungal Cultures: Ithaca, New York) and CBS (Fungal Biodiversity Center, Centraalbureau voor Schimmelcultures Fungal Biodiversity Center, Utrecht, The Netherlands) and represent a subset of the more than 200 isolates examined in the development of this study. These 57 isolates were selected because they were ex-types and/or because they represented the most inclusive phylogenetic sampling of the complex based on analysis of the 5′ end of EF-1α (tree not shown). Included among the isolates examined are ex-types for *Metarhizium anisopliae* var. *anisopliae* (ARSEF 7487, ex-neotype), *M. anisopliae* var. *acridum* (ARSEF 7486, ex-holotype; ARSEF 324, ex-paratype; ARSEF 3391, ex-paratype), *M. anisopliae* var. *lepidiotae* (ARSEF 7488, ex-paratype), *M. flavoviride* (ARSEF 2133, ex-holotype), *M. frigidum* (ARSEF 4124, ex-holotype), *M. guizhouense* (CBS 258.90, ex-holotype), *M. pingshaense* (CBS 257.90, ex-holotype) and that of two newly described species described herein (ARSEF 2596, ex-holotype; ARSEF 2575, ex-holotype). There is no viable ex-type culture for *M. brunneum* Petch. However ARSEF 2107 is considered an authentic strain because the taxon's author, Petch (1935), identified it



FIGS. 1–20. Mature conidiogenous cells and conidia of *Metarhizium* species. Bar = 10 µm. 1–2. *Metarhizium majus* (ARSEF 1914). 1. Phialides with developing conidia. 2. Mature conidia. 3–4. *M. guizhouense*. 3. Phialides with developing conidia (ARSEF 7507). 4. Mature conidia (ARSEF 6238). 5–6. *M. pingshaense*. 5. Phialides with developing conidia (ARSEF 4342). 6. Mature conidia (ARSEF CBS 257.90). 7–8. *M. anisopliae*. 7. Phialides with developing conidia (ARSEF 7487). 8. Mature conidia (ARSEF 7487). 9–10. *M. robertsii*. 9. Phialides with developing conidia (ARSEF 727). 10. Mature conidia (ARSEF 727). 11–12. *M. brunneum*. 11. Phialides with developing conidia (ARSEF 4179). 12. Mature conidia (ARSEF 2107). 13–14. *M. lepidiotae*. 13. Phialides with developing conidia (ARSEF 4628). 14. Mature conidia (ARSEF 7412). 15–16. *M. acridum*. 15. Phialides (ARSEF 6592); note that apices of conidiogenous cells thicken with successive conidial production. 16. Mature conidia (ARSEF 5736). 17–18. *M. globosum*. 17. Phialides (ARSEF 2596). 18. Mature conidia (ARSEF 2596). 19. *M. flavoviride*. Mature conidia (ARSEF 2025). 20. *M. frigidum*. Mature conidia (ARSEF 4124).

and we designate it here as an ex-epitype. The ex-holotype isolate of *M. anisopliae* var. *majus* (IMI 171404) is no longer viable (Aquino de Muro pers comm). However a broad geographic sampling of *M. anisopliae* var. *majus* was used to assess the status of this taxon and an ex-epitype (BPI 878297) derived from a living culture (ARSEF 1914) is designated for this taxon.

Morphological evaluations.—Isolates were grown under ambient light/dark conditions in the laboratory under fluorescent illumination at approximately 23 C on one-quarter strength SDAY (SDAY/4) (SDAY: 10 2.5 g/L Bactopeptone, 10 g/L dextrose, 2.5 g/L yeast extract, 20 g/L agar). Observations and measurements were made on 4–14 d old cultures with a Nikon Eclipse E600

compound microscope with differential interference contrast illumination. Images were captured with ACT-1 version 2.12 software (FIGS. 1–20, Nikon Corp.). Minimum and maximum values are shown (TABLE I) and are based on a minimum of 20 measurements for each feature.

DNA extraction.—Isolates were grown in SDY/4 broth 5–7 d on an orbital shaker set at 125 rpm and 25 C. The tissue was removed from the broth, rinsed twice with sterile water, blotted dry with filter paper, frozen at −80 C and lyophilized. Approximately 50 mg lyophilized mycelium was ground into powder with the FastPrep tissue homogenizer (MP Biomedicals, Irvine, California) for 6 s at a speed setting of 4.5. The pulverized tissue was lysed with 900 µL lysis buffer (2 M NaCl, 0.4% w/v deoxycholic acid, 1.0% w/v polyoxyethylene ether) and incubated 10 min at 55 C. Cellular byproducts were extracted with 750 µL chloroform : isoamyl alcohol (24 : 1) and centrifuged to separate the aqueous and particulate phases. The 700 µL of cleared solution containing DNA was removed, placed in a clean tube and mixed with an equal volume of 6 M guanidinium isothiocyanate. DNA was bound to 40 µL 50 : 50 suspension of acid-washed diatomaceous earth (Sigma, St Louis, Missouri) and flint glass powder (Minnesota Midwest Clay, Bloomington, Minnesota) prepared according Vogelstein and Gillespie (1979). The silica-bound DNA was suspended twice in 75% ethanol, dried, eluted in sterile distilled water by incubating 5 min at 55 C.

PCR amplification and nucleotide sequencing.—Genes used in this study were translation elongation factor 1-alpha (EF-1α), RNA polymerase II largest subunit (RPB1), RNA polymerase second largest subunit (RPB2), β-tubulin (Bt) and the nuclear ribosomal intergenic spacer region (IGS). Only the 5′ end of EF-1α was sequenced for all 57 isolates. Also, using the 5′ end of EF-1α we determined the phylogenetic placement of two presumptive cryptic species of *M. anisopliae* reported by Bidochka et al (2001, 2005). Partial sequences of EF-1α, RPB1, RPB2 and Bt were sequenced for a subset of 33 isolates for the combined multigene analysis. In addition to the primers used in Bischoff et al (2006) and the Bt primers T1 and T22 as described by O'Donnell and Cigelnik (1997) these primers were developed for sequencing purposes: RPB1: Mz1F1 (5′-CGRACMYTRCCYCATTTCACAA), Mz1R1 (5′-TTGAGCGGAAGYTGCATCATCTCC), Mz1R2(5′-TTCARRAARGCCATSGCRCCWTC); Bt: Bt1F (5′-GGTCCCTTCGGTCAGCTCTTCC), Bt1R (5′-CAGCCATCATGTTCTTAGGGTC), Bt2R (5′-GTAGTGACCCTTGGCCCAGT).

IGS was sequenced for exemplars validated in this study for these species: *M. anisopliae* (ARSEF 7450, ARSEF 7487), *M. brunneum* (ARSEF 2107), *M. guizhouense* (ARSEF 4303, ARSEF 4321, ARSEF 6238, ARSEF 7502, ARSEF 7507, CBS 258.90), *M. majus* (ARSEF 1015, ARSEF 1914, ARSEF 1946, ARSEF 2808, ARSEF 4566, ARSEF 7505), *M. pingshaense* (ARSEF 3210, ARSEF 4342, ARSEF 7929, CBS 257.90) and *M. robertsii* sp. nov. (ARSEF 727, ARSEF 2575). IGS, including partial segments of the flanking large and small ribosomal subunits, was amplified in two overlapping segments, designated here as IGSa and IGSb. IGSa was amplified with primer pairs LSU4 (5′-CCGTYCTTCGCCYCGATTTCC) and Ma-IGS1 (5′-CGTCACTTGTATTGGCAC, Pantou et al 2003) and primer pairs 630 U (5′-CTTTAGGGTAGGCTGCTTGTT) and NS2 (5′-GGCTGCTGGCACCAGACTTGC, White et al 1990). Internal primers were developed for sequencing IGSa (IGSaF12: 5′-GTACCCGGGACYCCRAGTAAG; IGSbR2: 5′-GYTCCTGGTCGGGACTTAYA) and IGSb (IGSbF4: 5′-GCGYGYWGWATTRRATGGTYT; IGSaR4: 5′-ACCGGGCGCTCGTGKTCYATT) amplicons. All loci were amplified and sequenced according to Bischoff et al (2006).

Sequence alignment and phylogenetic analyses.—Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan) was used to assemble and edit sequence data. Alignments were made with Clustal X (Thompson et al 1997) with the default settings. Adjustment to the computer-assisted alignment was needed only in the case of the intron-containing 5′ region of EF-1α. These adjustments were made by eye and involved the elimination of ambiguous regions created by insertions and deletions (indels).

Phylogenetic hypotheses were developed with maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods. The MP analyses were performed with PAUP* 4.0b10 (Swofford 2002) with heuristic searches of 500 random-addition replicates with TBR branch-swapping and equal character weighting. Heuristic MP bootstrap (MP BS) analyses (Felsenstein 1985) with TBR branch-swapping included 1000 pseudoreplicates, and 10 random addition replicates were performed to provide bootstrap support values. The program GARLI 0.95 (Zwickl 2006) was used to identify the most likely tree and to determine maximum likelihood bootstrap support (ML BS). The default settings in GARLI were used, and 1000 bootstrap repetitions were performed. All log-likelihood (−lnL) scores reported were calculated with PAUP.

MrBayes 3.1 (Huelsenbeck 2000, Ronquist and Huelsenbeck 2003) was used for Bayesian analyses to determine posterior probabilities (BI PP). The analysis was run four times with each run including 4 MCMC chains (three cold, one heated), 1 000 000 generations, sampling every 100 generations (including the first generation) for a total of 40 004 trees. The first 20% of trees from each run were discarded to allow the log-likelihood scores to achieve stationarity (i.e. burn-in). The resulting trees were imported into PAUP, and a 50% consensus tree was computed.

Clades that are supported with 95% BI PP or greater, 70% ML BS or greater and 70% MP BS or greater values were considered significantly supported by the data (Mason-Gamer and Kellogg 1996, Lutzoni et al 2004, Reeb et al 2004). Mason-Gamer and Kellog (1996), Lutzoni et al (2004) and Reeb et al (2004) did not address a threshold criterion for determining significant support based on ML BS values. GARLI was not yet available when these two papers were published. However because the ML BS support values from the GARLI analysis in this study were close in absolute value to those from the MP BS analysis we elected to use a 70% threshold to identify significantly supported data for ML BS values. The phylograms (FIGS. 21, 22) are the consensus trees with the mean branch lengths from the Bayesian analyses.

TABLE I. Strain codes, taxon name, host, country of collection, GenBank accession numbers, conidial measurements and color in culture in SDAY/4.

Voucher #	Taxon	Isolation source	Country	GenBank accession numbers					Conidia (μm)	Phialides (μm)	Color code	Color description
				EF1-alpha	EF1-5′	RPB1	RPB2	Beta-tub				
473	*Metarhizium majus*	Soil	Australia		EU248875				9.5–12.0 × 2.5–4.0	11.0–23.5 × 2.5–3.5		Olive
978	*Metarhizium majus*	Coleoptera	France		EU248889							
1015	*Metarhizium majus*	Lepidoptera	Japan	EU248866	EU248866	EU248918	EU248946	EU248838	10.0–14.5 × 2.5–4.0	10.0–17.0 × 2.5–4.0		Olive
1858	*Metarhizium majus*	Coleoptera	Poland		EU248872				10.0–14.5 × 2.5–4.0			Olive
1914†	*Metarhizium majus*	Coleoptera	Philippines	EU248868	EU248868	EU248920	EU248948	EU248840	10.5–13.0 × 2.5–4.0	10.0–18.0 × 2.5–3.5	1F4	Olive
1946	*Metarhizium majus*	Coleoptera	Philippines	EU248867	EU248867	EU248919	EU248947	EU248839	10.5–13.5 × 3.0–4.0	12.5–16.5 × 3.0–4.0	29F3	Dark green
2808	*Metarhizium majus*	Coleoptera	Philippines	EU248871	EU248871	EU248923	EU248951	EU248843				
4566	*Metarhizium majus*	Coleoptera	Australia	EU248869	EU248869	EU248921	EU248949	EU248841				
7505	*Metarhizium majus*	Coleoptera	Australia	EU248870	EU248870	EU248922	EU248950	EU248842	8.5–13.0 × 3.0–5.0	9.0–18.5 × 2.5–4.5	1E4	Olive
4303	*Metarhizium guizhouense*	Soil	Australia	EU248859	EU248859	EU248911	EU248939					
4321	*Metarhizium guizhouense*	Soil	Australia	EU248860	EU248860	EU248912	EU248940	EU248832	5.5–8.0 × 2.5–3.5	8.0–12.0 × 2.5–3.5	1F7	Olive
4604	*Metarhizium guizhouense*	Coleoptera	Papua New Guinea		EU248894							
5714	*Metarhizium guizhouense*			EU248856	EU248856	EU248908	EU248936	EU248829	7.0–9.0 × 2.0–3.0	7.5–20.0 × 2.0–3.0		Olive
6238	*Metarhizium guizhouense*	Lepidoptera	China	EU248857	EU248857	EU248909	EU248937	EU248830	6.5–8.0 × 2.5–3.0	7.0–12.0 × 2.0–3.0		Olive
7420	*Metarhizium guizhouense*	Diptera	Australia		EU248892							
7502	*Metarhizium guizhouense*		Australia	EU248861	EU248861	EU248913	EU248941	EU248833	7.0–8.5 × 2.5–3.5	6.0–12.0 × 2.0–3.5		Olive
7507	*Metarhizium guizhouense*	Soil	Kiribati	EU248858	EU248858	EU248910	EU248938	EU248831	6.5–8.5 × 2.0–3.0	7.5–13.5 × 2.0–3.0	2F6	Olive
CBS 258.90*	*Metarhizium guizhouense*	Lepidoptera	China	EU248862	EU248862	EU248914	EU248942	EU248834	7.0–10.0 × 2.0–3.0	7.0–12.0 × 2.0–3.0	1F4	Olive
3210	*Metarhizium pingshaense*	Coleoptera	India	DQ463995	DQ463995	DQ468354	DQ468369	EU248819			2F7	Olive

TABLE I. Continued

Voucher #	Taxon	Isolation source	Country	GenBank accession numbers					Conidia (μm)	Phialides (μm)	Color code	Color description
				EF1-alpha	EF1-5′	RPB1	RPB2	Beta-tub				
4342	*Metarhizium pingshaense*	Coleoptera	Solomon Islands	EU248851	EU248851	EU248903	EU248931	EU248821	4.5–7.0 × 2.0–3.0	7.0–13.0 × 2.0–3.0	2D4	Olive
7929	*Metarhizium pingshaense*	Isoptera	Australia	EU248847	EU248847	EU248899	EU248927	EU248815				
CBS 257.90*	*Metarhizium pingshaense*	Coleoptera	China	EU248850	EU248850	EU248902	EU248930	EU248820	6.0–8.0 × 2.5–3.5	7.0–17.0 × 2.5–3.5		Olive
727	*Metarhizium robertsii*	Orthoptera	Brazil	DQ463994	DQ463994	DQ468353	DQ468368	EU248816	5.0–7.0 × 2.0–3.5	7.0–14.5 × 2.5–3.5	1D7	Grayish-green
4739	*Metarhizium robertsii*	Soil	Australia	EU248848	EU248848	EU248900	EU248928	EU248817			1F6	Olive
6472	*Metarhizium robertsii*	Coleoptera	USA		EU248884							
7501	*Metarhizium robertsii*		Australia	EU248849	EU248849	EU248901	EU248929	EU248818	5.0–7.5 × 2.0–3.0	6.0–12.0 × 2.0–3.0		Olive
6347	*Metarhizium anisopliae*	Hemiptera	Colombia		EU248881							
7450	*Metarhizium anisopliae*	Coleoptera	Australia	EU248852	EU248852	EU248904	EU248932	EU248823				
7487*	*Metarhizium anisopliae*	Orthoptera	Eritrea	DQ463996	DQ463996	DQ468355	DQ468370	EU248822	5.0–7.0 × 2.0–3.5	8.0–11.5 × 2.0–3.0	1D6	Grayish-green
988	*Metarhizium brunneum*	Lepidoptera	Japan		EU248890							
2107†	*Metarhizium brunneum*	Coleoptera	USA	EU248855	EU248855	EU248907	EU248935	EU248826	4.5–7.5 × 2.0–3.0	9.5–18.0 × 2.0–5.0	2A3	Pale yellow
3826	*Metarhizium brunneum*	Diptera	USA		EU248874				5.0–6.5 × 2.0–3.0	7.0–10.5 × 2.0–3.0		Olive
4152	*Metarhizium brunneum*	Soil	Australia	EU248853	EU248853	EU248905	EU248933	EU248824	5.0–7.0 × 2.5–3.5	7.5–13.5 × 2.5–4.0	1E7	Olive
4179	*Metarhizium brunneum*	Soil	Australia	EU248854	EU248854	EU248906	EU248934	EU248825	5.5–8.0 × 2.0–3.0	6.0–13.0 × 2.0–3.0	2F6	Olive
5198	*Metarhizium brunneum*	Coleoptera	Germany		EU248876							
5625	*Metarhizium brunneum*	Coleoptera (baited)	Finland		EU248877							
6120	*Metarhizium brunneum*	Coleoptera	Argentina		EU248880							

TABLE I. Continued

Voucher #	Taxon	Isolation source	Country	GenBank accession numbers					Conidia (μm)	Phialides (μm)	Color code	Color description
				EF1-alpha	EF1-5′	RPB1	RPB2	Beta-tub				
6392	*Metarhizium brunneum*	Coleoptera	USA		EU248882							
6477	*Metarhizium brunneum*	Coleoptera	Norway		EU248885							
4154	*Metarhizium lepidiotae*	Soil	Australia		EU248891				6.0–7.5 × 2.0–3.0	6.5–12.0 × 2.0–3.0	1F6	Olive
4587	*Metarhizium lepidiotae*	Coleoptera or Hemiptera	Papua New Guinea		EU248893							
4628	*Metarhizium lepidiotae*	Soil	Australia	EU248863	EU248863	EU248915	EU248943	EU248835	6.0–7.5 × 2.5–3.5	7.0–12.0 × 2.0–3.0		Olive
4660	*Metarhizium lepidiotae*	Coleoptera or Hemiptera	Papua New Guinea		EU248895							
7412	*Metarhizium lepidiotae*	Coleoptera	Australia	EU248864	EU248864	EU248916	EU248944	EU248836	5.0–7.0 × 2.0–3.0	7.0–10.5 × 2.0–3.0	1F7	Olive
7488*	*Metarhizium lepidiotae*	Coleoptera	Australia	EU248865	EU248865	EU248917	EU248945	EU248837	5.0–7.0 × 3.0–4.0	8.0–12.0 × 2.5–3.5	1E4	Olive
324	*Metarhizium acridum*	Orthoptera	Australia	EU248844	EU248844	EU248896	EU248924	EU248812	5.0–7.5 × 3.0–4.5		2B6	Grayish-yellow
3391	*Metarhizium acridum*	Orthoptera	Tanzania		EU248873				4.5–5.5 × 2.5–3.5			
5736	*Metarhizium acridum*	Orthoptera	Madagascar		EU248878				4.5–5.5 × 2.0–3.5			
5748	*Metarhizium acridum*	Orthoptera	Mexico		EU248879				4.0–5.0 × 2.5–3.5	6.0–13.0 × 2.0–3.5		Grayish-green
6421	*Metarhizium acridum*	Orthoptera	Senegal		EU248883							
6592	*Metarhizium acridum*	Abandoned termite mound	Benin		EU248886				4.0–5.5 × 2.0–3.0	5.0–11.5 × 2.0–3.0	1B5	Grayish-yellow
6597	*Metarhizium acridum*	Soil	Benin		EU248887							
6600	*Metarhizium acridum*		Benin		EU248888				4.0–5.5 × 2.0–3.0			Grayish-green
7486*	*Metarhizium acridum*	Orthoptera	Niger	EU248845	EU248845	EU248897	EU248925	EU248813	4.0–5.5 × 3.0–4.0	4.5–12.5 × 2.5–4.5	1B5	Grayish-yellow

TABLE I. Continued

Voucher #	Taxon	Isolation source	Country	GenBank accession numbers					Conidia (µm)	Phialides (µm)	Color code	Color description
				EF1-alpha	EF1-5′	RPB1	RPB2	Beta-tub				
2596*	*Metarhizium globosum*	Lepidoptera	India	EU248846	EU248846	EU248898	EU248926	EU248814	4.0–5.0 × 4.0–5.0	5.0–12.0 × 3.0–4.0	27C6	Grayish-green
2133*	*Metarhizium flavoviride*	Coleoptera	Czech Rep.	DQ463999	DQ463988	DQ468358	DQ468373	EU248827				
4124*	*Metarhizium frigidum*	Coleoptera	Australia	DQ464002	DQ463978	DQ468361	DQ468376	EU248828	4.5–7.5 × 2.5–3.5		28E7	Bright green

All strains are from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) with the exception of those prefixed with CBS (Centraalbureau voor Schimmelcultures, Netherlands). Sequences and morphological data are not available for all strains (i.e. blank cells). Color codes and descriptions are based on the Methuen Handbook of Colour, Second Edition (Kornerup & Wanscher 1967).

* Denotes an ex-type isolate.

† Denotes an ex-epitype designated in this study.

EF-1α, RPB1, RPB2 and Bt first were analyzed independently with MP and ML BS to identify areas of concordance and discordance among the phylogenies of the genes sampled (Dettman et al 2003) and to compare bootstrap support for clades resolved in the different gene trees. In addition, to broaden the scope of taxon sampling and to focus on the most recently diverged lineages, the rapidly evolving, intron-rich region of the 5′ end of EF-1α was analyzed independently (see FIG. 22).

The results of Driver et al (2000), Spatafora et al (2007), Sung et al (2007) and our own unpublished work have confirmed that *Metarhizium* is a monophyletic group. All phylogenetic analyses of the *M. anisopliae* complex in this study used the closely related species *M. flavoviride* and *M. frigidum* as outgroup taxa.

TAXONOMY

Based on the results of the phylogenetic analyses and the morphological data, we describe two new species, elevate three varieties of *M. anisopliae* to species level, resurrect the taxon *M. brunneum* and recognize *M. guizhouense* as the anamorph of *Metacordyceps taii*. Despite our inability to delimit all these taxa based on morphological characteristics, the molecular data support the recognition of these fungi at the species level.

Metarhizium acridum (Driver & Milner) J.F. Bisch., Rehner & Humber stat. nov. FIGS. 15–16

MycoBank MB512407

≡ *Metarhizium anisopliae* var. *acridum* Driver & Milner, Mycol. Res. 104:144 (2000).

Metarhizium anisopliae (Metsch.) Sorok., Plant parasites of man and animals as causes of infectious diseases 2:268 (1883). FIGS. 7–8

≡ *Entomophthora anisopliae* Metsch. Zapiski imperatorskogo obshchestua sel'ska Khozyaistra yuzhnoi rossii p 45 (1879).

≡ *Isaria anisopliae* (Metsch.) Pettit, Cornell Univ. Agric. Exp. St. Bull. 97:356 (1895).

≡ *Penicillium anisopliae* (Metsch.) Vuill., Bull. Trimest. Soc. Mycol. Fr. 20:221 (1904).

= *Isaria destructor* Metsch., Zool. Anz. 3:45 (1880).

= *Oospora destructor* (Metsch.) Delacroix, Bull. Trimest. Soc. Mycol. Fr. 9:260 (1893).

= *Isaria anisopliae* var. *americana* Pettit, Cornell Univ. Agric. Exp. St. Bull. 97:354 (1895)

= *Penicillium cicadinum* Höhn., Sber. Akad. Wiss. Wien 118:405 (1909).

= *Metarhizium cicadinum* (Höhn.) Petch, Trans. Br. Mycol. Soc. 16:68 (1931).

= *Sporotrichum paranense* Marchionatto, Bol. Mens. Min. Agric. Noac. Buenos Aires 34:241 (1933).

TABLE II. Nodal support values for *Metarhizium* species lineages from GARLI analyses of individual genes. Values in parentheses indicate support for conflicting topology (see Results for details)

	EF-1α	RPB1	RPB2	B-tubulin
M. acridum	100	100	100	99
M. anisopliae	100	98	100	100
M. brunneum	100	100	—	—
M. globosum	N/A	N/A	N/A	N/A
M. guizhouense	89	—	(95)	—
M. lepidiotae	—	100	98	—
M. majus	96	—	(99)	—
M. pingshaense	82	—	—	100
M. robertsii	100	—	—	99

Metarhizium brunneum Petch, Trans. Br. Mycol. Soc. 19:189 (1935). FIGS. 11–12

In the absence of a readily accessible type for *Metarhizium brunneum*, we designate a dried culture stored at the US National Fungus Collection (BPI 878293) as an epitype. This epitype was prepared from a plate of the isolate ARSEF 2107, which also is deposited elsewhere as CBS 316.51, IMI 014746, NRRL1944 and QM 191.

Metarhizium globosum J.F. Bisch., Rehner & Humber sp. nov. FIGS. 17–18

Coloniae primum albae, transeuns griseolus-viridus. Hyphae vegetativae 2.0–4.0 µm crasse. Conidiophorum erectus. Phialides clavatus, 5.0–12.0 µm longae et 3.0–4.0 µm crasse. Conidia globosus, 4.0–5.0 µm crasse.

Colony grown at 25 C on SDY/4 medium is first white becoming pigmented within 5 d to a grayish-green (color plate 27C6, Kornerup and Wanscher 1967). Vegetative hyphae are smooth and 2.0–4.0 µm. Conidiophores are erect, terminating in branches with 2–3 phialides per branch, forming a palisade-like layer. Phialides (FIG. 15) are clavate, 5.0–12.0 µm long and 3.0–4.0 µm at their broadest point with strongly tapering apices. Conidia are globose, 4.0–5.0 µm diam.

Typus: INDIA. On *Pyrausta machaeralis* (Lepidioptera, Pyralidae), 12 Sep 1988, collected by *RC Rajak* (*ENT/12*) (HOLOTYPE BPI 878294 [dried culture]; ARSEF 2596 ex-type).

Etymology: *M. globosum* is named for its globose conidia.

Metarhizium guizhouense Q.T. Chen & H.L. Guo, Acta Mycologica Sinica 5:181 (1986). FIGS. 3–4

≡ *Metarhizium taii* Z.Q. Liang & A. Y. Liu, Acta Mycologica Sinica 10:260 (1991).

Teleomorph: *Metacordyceps taii* (Z.Q. Liang & A.Y. Liu) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, Acta Mycologica Sinica 10:257 (1991).

Metarhizium lepidiotae (Driver & Milner) J.F. Bisch., Rehner & Humber stat. nov. FIGS. 13–14

MycoBank MB512409

≡ *Metarhizium anisopliae* var. *lepidiotae* Driver & Milner (as *Metarhizium anisopliae* var. *lepidiotum*), Mycol. Res. 104:145 (2000).

Metarhizium majus (J.R. Johnst.) J.F. Bisch., Rehner & Humber stat. nov. FIGS. 1–2

MycoBank MB512410

≡ *Metarhizium anisopliae* f. *major* J.R. Johnst., Entomogenous Fungi of Porto Rico p 27 (1915).

= *Metarhizium anisopliae* f. *oryctophagum* Friederichs, Die Grundfragen und Gesetzmassigkeiten der Land-und Forstwirtschäflichen Zoologie p 199 (1930).

≡ *Metarhizium anisopliae* var. *major* (J.R. Johnst.) M.C. Tulloch, Trans. Brit. Mycol. Soc. 66:409 (1976).

In the absence of a useful type for *Metarhizium majus* we designate a dried culture stored at the US National Fungus Collection (BPI 878297) as an epitype. This epitype was prepared from a plate of the isolate ARSEF 1914.

Metarhizium pingshaense Q.T. Chen & H.L. Guo, Acta Mycologica Sinica 5:181 (1986). FIGS. 5–6

Metarhizium robertsii J.F. Bisch., Rehner & Humber sp. nov. FIGS. 9–10

MycoBank MB512411

Anamorphus in morphologia idem ac *Metarhizium anisopliae*, sed distinguibilis characteribus sequentibus nucleotiditis fixationibus: translation elongation factor positions 257 (gap), 258 (T), 287 (A), 447 (G) and 806 (C).

Metarhizium robertsii is morphologically indistinguishable from *M. anisopliae*. It is diagnosed from other members of the *M. anisopliae* complex by these unique fixed nucleotide characters in the EF-1α positions: 257 (gap), 258 (T), 287 (A), 447 (G) and 806 (C). Nucleotide positions can be evaluated by downloading the alignment S2169 from www.TreeBase.org.

Typus: USA. SOUTH CAROLINA, on *Curculio caryae* (Coleoptera, Curculionidae) (holotype BPI 878819 is a specimen derived from ARSEF 2575 ex-type).

Etymology: *M. robertsii* is named in honor of Donald W. Roberts.

Isolate ARSEF 2575 was selected as the ex-type of *M. robertsii* because it is widely recognized as an important isolate in insect biocontrol. Although this strain was not included in the phylogenetic trees presented the genes EF-1α, Bt, and RPB1 were amplified and analyzed and found to be well supported within the *Metarhizium robertsii* clade. Furthermore ARSEF 2575 was found to exhibit the

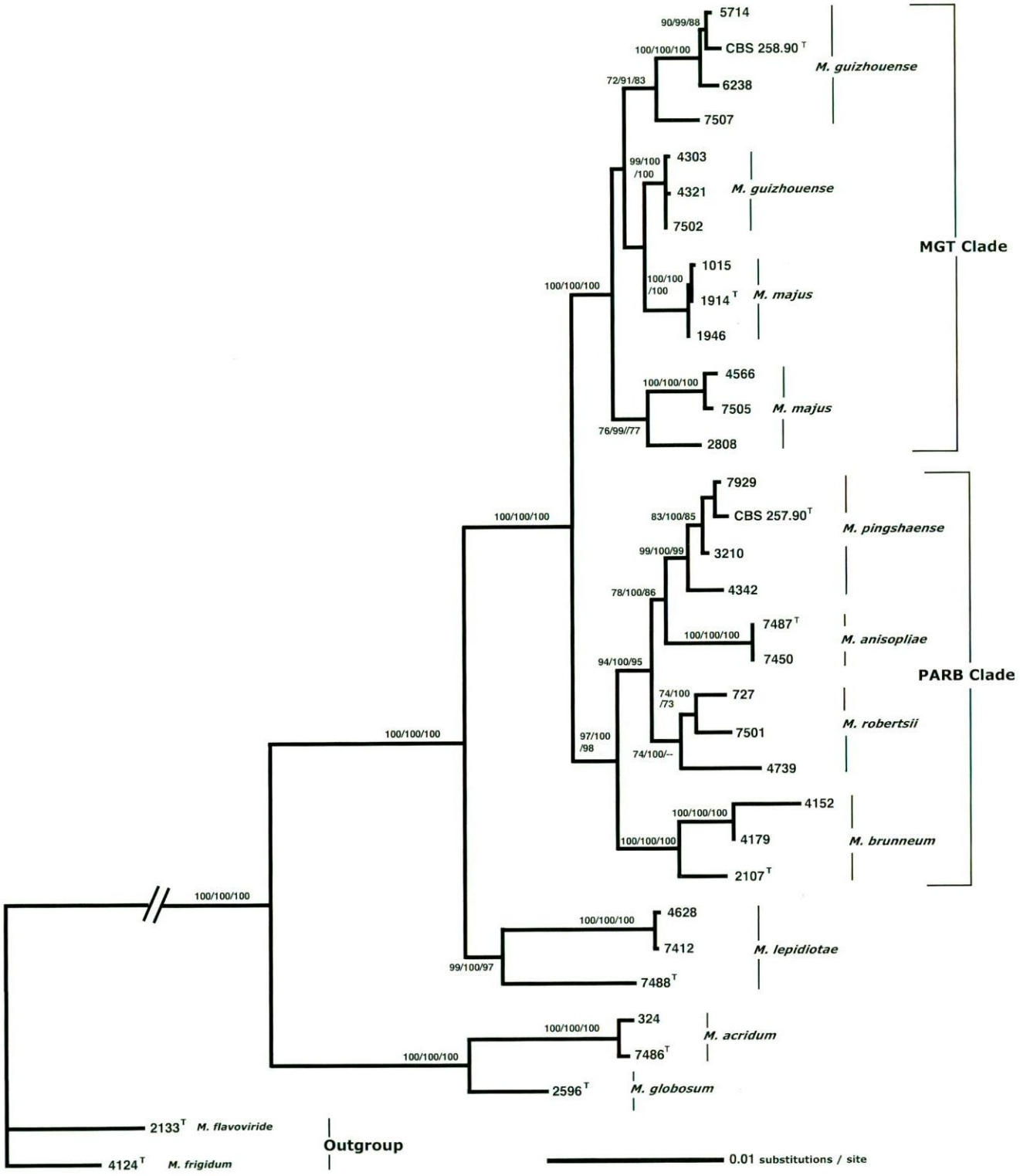


FIG. 21. Majority rule consensus phylogram from the Bayesian combined analysis of EF-1α, RPB1, RPB2, Bt (−lnL 19284.18). Support values greater than 70% are shown for ML BS, BI PP and MP BS respectively (– = support value < 70%). Ex-type isolates are denoted with a "T".

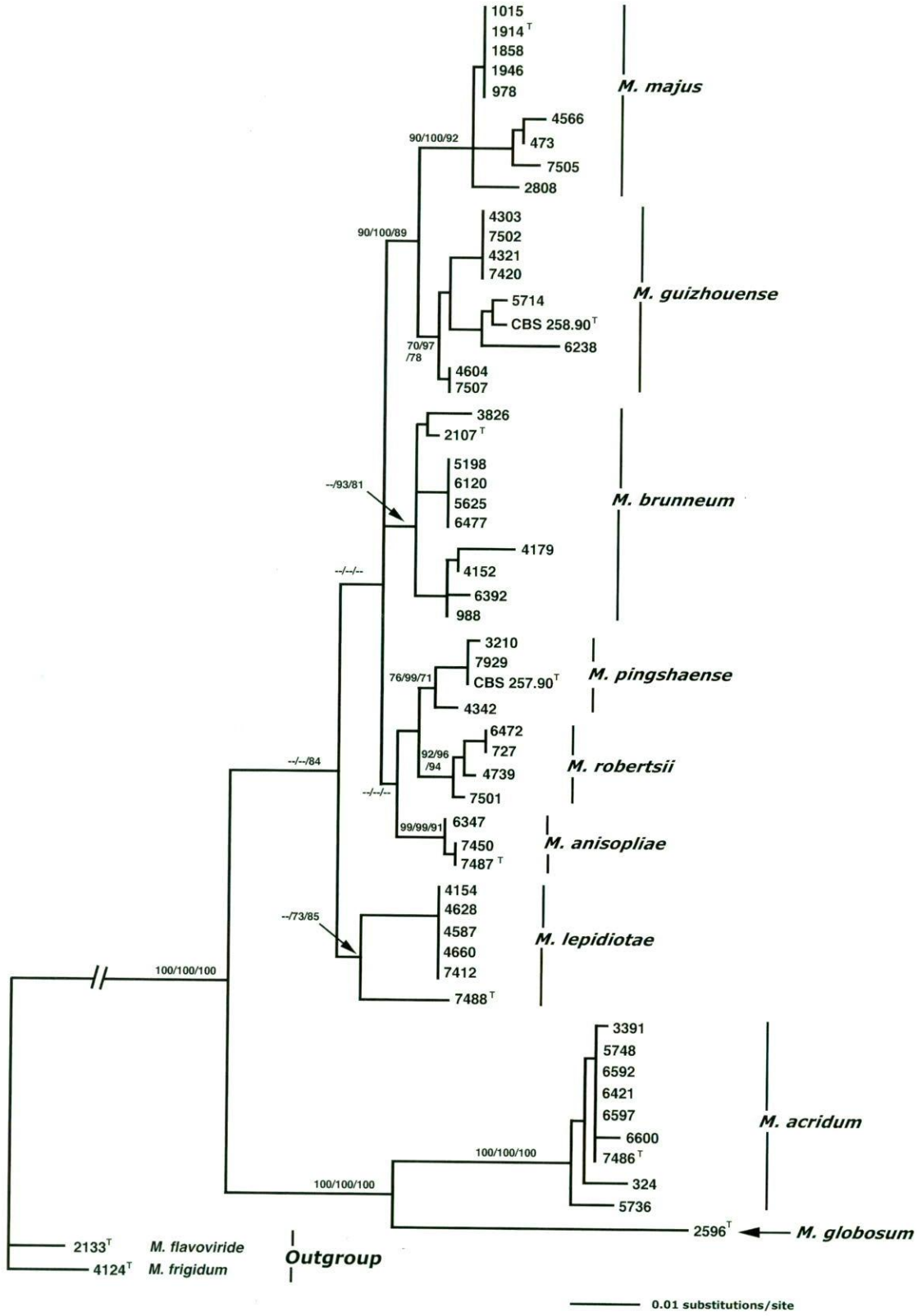


FIG. 22. Majority rule consensus phylogram from the Bayesian analysis of the 5′ end of EF-1α for 57 isolates (−lnL 2560.26). Support values greater than 70% are shown for ML BS, PP and MP BS respectively (– = support value < 70%). Support values are included for the backbone and terminal taxa nodes only to prevent overcrowding the figure. Ex-type isolates are denoted with a "T".

same informative EF-1α nucleotide bases used to distinguish this species from other members of the *M. anisopliae* complex. A single-spore isolate of ARSEF 2575, accessioned as ARSEF 8020, has been designated for full genome sequencing (D. Gibson pers comm).

Dong et al (2007) introduced the name *Metarhizium anisopliae* "var. *dcjhyium*". Their paper did not include a description or holotype designation and therefore this taxon is an invalidly published nomen nudum. Furthermore Dong et al (2007) provided no morphological evidence nor did they evaluate *M. anisopliae* "var. *dcjhyium*" in the context of other *M. anisopliae* varieties. No isolates associated with the name *M. anisopliae* "var. *dcjhyium*" are available for study at this time, and we reject this taxon due to its invalid nomenclatural status and lack of any morphological or molecular evidence that distinguishes it from other species of the genus.

RESULTS

Phylogenetic analyses.—Sequencing of the four nuclear genes from an exemplar set of 33 *Metarhizium* isolates, after exclusion of 104 ambiguously aligned sequence positions, yielded a total of 7621 aligned characters that included a total of 741 parsimony informative characters: 1700 bp for EF-1α (163 parsimony informative characters), 2783 bp for RPB1 (227 parsimony informative characters), 1799 bp for RPB2 (201 parsimony informative characters), 1339 bp for Bt (150 parsimony informative characters). In a separate analysis of the 5′ end of EF-1α for an expanded set of 57 isolates the aligned length of this locus was 681 bp (139 parsimony informative characters). Alignments are available through www.TreeBASE.org (accession number S2169).

Preliminary GARLI ML analyses of the individual genes were conducted to assess whether the inferred tree topologies from the different data partitions were phylogenetically congruent. These analyses yielded significant support from at least two genes for seven of the nine terminal clades that we interpret to constitute phylogenetic species (single-gene trees not shown). Bootstrap support values for the nine species recognized are provided (TABLE II). Topological conflict among the gene trees was confined to the clade containing *M. majus* and *M. guizhouense* isolates and is discussed below.

A clade containing the ex-neotype isolate of *M. anisopliae*, ARSEF 7487, is designated here as the PARB clade and consists of four subclades that we recognize as the species *M. anisopliae*, *M. pingshaense*, *M. robertsii* and *M. brunneum*. *M. anisopliae* received significant support from all four gene partitions and conflicted in none (TABLE II). The monophyly of both *M. pingshaense* and *M. robertsii* received significant support from both EF-1α and Bt and did not conflict with either the RPB1 or RPB2 topologies. The monophyly of *M. brunneum*, which is the most basal lineage of the PARB clade, received significant support from EF-1α and RPB1 and conflicted in none.

The single instance of significant conflict among the individual gene analyses occurred in the placement and relationships of isolates of *M. majus* and *M. guizhouense* (= *M. taii*), henceforth referred to as the MGT clade. EF-1α was the only gene phylogeny that significantly supported the reciprocal monophyly of *M. majus* and *M. guizhouense* (96% and 89% ML BS respectively). RPB2 conflicted with the results of the EF-1α analysis in that *M. majus* isolates ARSEF 4566, ARSEF 7505 and ARSEF 2808 were placed in a clade along with the ex-type of *M. guizhouense* (CBS 258.90), ARSEF 7507, ARSEF 5714 and ARSEF 6238 (95% ML BS), while the other isolates of *M. majus* (ARSEF 1015, 1946 and 1914) and *M. guizhouense* (ARSEF 4303, 4321 and 7502) were sister clades (99% ML BS). The relationships among the *M. majus* and *M. guizhouense* isolates in the Bt analysis conflicted with both the EF-1α and RPB2 analyses by grouping *M. majus* isolates ARSEF 4566, 7505 and 2808 in a single distinct clade (94% ML BS) and all other *M. majus* and *M. guizhouense* isolates as a sister clade (81% ML BS). The ex-holotype of *M. guizhouense*, CBS 258.90, was placed in the same clade as the available *M. taii* isolates (ARSEF 5714 and ARSEF 6238) in each of the single gene phylogenies, thus suggesting their synonymy as is discussed further below.

The monophyly of *M. lepidiotae* was supported concordantly by RPB1 and RPB2 and did not conflict with either EF-1α or Bt. *Metarhizium acridum* was significantly supported by all four gene partitions. However the basal lineage of the *M. acridum* clade, ARSEF 2596, was phylogenetically distinct in all analyses, differed morphologically from other *M. acridum* isolates and is described herein as the new species *M. globosum*. ARSEF 2596 is at present the only isolate available for *M. globosum*, thus statistical support for this lineage cannot be determined. Because topological conflict among individual gene trees was limited to sister lineages within a single derived lineage, the MGT clade, we opted to combine the datasets (FIG. 21).

In the combined dataset analyses the consensus tree with mean branch lengths as determined by MrBayes (−lnL 19284.18, FIG. 21) and the best tree retrieved from the ML GARLI analysis (−lnL 19284.18) were topologically identical. The combined

gene tree topology was consistent with the topologies inferred from analyses of individual genes. The MP BS tree (not shown) did not significantly conflict with either the BI or ML trees. The PARB clade (97% ML BS, 100% BI PP and 98% MP BS) comprises four species lineages, including *Metarhizium anisopliae* (100% ML BS, 100% BI PP and 100% MP BS), *M. pingshaense* (99% ML BS, 100% BI PP and 99% MP BS), *M. robertsii* sp. nov. (74 ML BS, 100% BI PP, 62% MP BS) and the most basal species lineage, *M. brunneum* (74% ML BS, 100% BI PP and 100% MP BS). The MGT clade (100% ML BS, 100% BI PP and 100% MP BS) was highly supported as the sister of the *M. anisopliae* clade (100% ML BS, 100% BI PP and 100% MP BS). However both species in this clade, *M. majus* and *M. guizhouense*, were paraphyletic with each forming two subclades in the combined gene analysis (FIG. 21). *Metarhizium lepidiotae* (99% ML BS, 100% BI PP and 97% MP BS) is strongly supported as the sister of the PARB and MGT clades (100 ML BS, 100% BI PP, 100% MP BS). The most basal clade in the *M. anisopliae* complex is that containing *M. acridum* and *M. globosum* (100% ML BS, 100% BI PP and 100% MP BS) with *M. acridum* strongly supported (100 ML BS, 100% BI PP, 100% MP BS) as distinct from *M. globosum*.

We further evaluated the relationships within the *M. anisopliae* complex by expanding the taxon set to 57 isolates, focusing exclusively on the 5′ end of EF-1α (FIG. 22). We selected this region for further analysis because, among the four loci analyzed in this study, the introns within 5′ EF-1α provide the greatest concentration of informative nucleotide variation and degree of phylogenetic resolution for terminal clades in *Metarhizium* (pers obs). In this analysis the consensus tree with mean branch lengths inferred with MrBayes (−ln L 2560.26, FIG. 22) and the best tree from the ML GARLI analysis (−ln L 2560.26) had identical topologies. The topology of the inferred 5′ EF-1α tree is consistent with the multigene phylogeny (FIG. 21), although nodal support values, particularly for internal nodes, are generally lower. This finding is not surprising given the fewer number of variable characters provided by 5′ EF-1α. However 5′ EF-1α alone provides strong support for all species lineages in this study and thus is the most accurate and expedient locus for use in species identification. A unique and notable result of the 5′ EF-1α phylogeny is that in the MGT clade (90% ML BS, 100% BI PP, 89% MP BS) *Metarhizium majus* and *M. guizhouense* are significantly supported as reciprocally monophyletic (90% ML BS, 100% BI PP, 92% MP BS and 70% ML BS, 97% BI PP, 78% MP BS respectively).

Two *M. anisopliae* s.l. isolates from Bidochka et al (2001, 2005), each representing one of the two clades identified as "cold-active"(43A-2i) and "heat-active"(MAA1-2ii) were obtained from the author and sequenced for the 5′ region of EF-1α only. Based on phylogenetic analysis, these isolates belong to *M. robertsii* and *M. brunneum* respectively (data not shown). Cultures and sequence accessions for these isolates were deposited in the ARSEF collection and GenBank (43A-2i: ARSEF 8680, FJ229493; MAA1-2ii: ARSEF 8685, FJ229494 respectively).

In addition, in a characterization of rDNA and associated spacer regions for *M. anisopliae* Pantou et al (2003) resolved three well supported clades, A–C, with IGS nucleotide sequences. IGS sequences were determined for 21 isolates (GenBank accessions FJ228703–FJ228723) from the PARB and MGT clades from the present study and compared to the data of Pantou et al (data not shown). These comparisons revealed that IGS Clade A includes *M. anisopliae*, *M. pingshaense* and *M. robertsii*. Within IGS Clade A *M. anisopliae* and *M. pingshaense* plus *M. robertsii* formed well supported reciprocally monophyletic groups; however the relationship of *M. pingshaense* and *M. robertsii* is unresolved, which form a paraphyletic grade. IGS Clade B corresponds to *M. brunneum*. IGS Clade C of Pantou et al (2003) is most closely related to *M. guizhouense* Clade 1; however in the limited sampling of IGS from isolates in this study the MGT clade is paraphyletic, in agreement with the results obtained for RPB1, RPB2 and Bt. Based on the present assessment, IGS by itself does not clearly resolve relationships in either the PARB or MGT clades.

Morphological observations.—Colony pigmentation of the various isolates of *Metarhizium anisopliae* complex is initially white and usually becomes yellow during the early development of conidia (typically 4–7 d) and becomes greenish as the conidia mature (see TABLE I). Yellow pigmentation is particularly conspicuous in *M. acridum*, which tends to retain its color 10–14 d. In addition we observed variation in colony color among the isolates within supported lineages that overlapped with color variation in other lineages, making it impossible to reliably assign diagnostic colors to taxonomically defined groups. In general the mature colony color of isolates from the *M. anisopliae* complex is best described as olivaceous or some slight variation of olive. Most isolates reached maturity 8–9 d. Likewise all examined members of the *M. brunneum* clade were olivaceous at maturity except for ARSEF 2107, which was pale yellow. *Metarhizium globosum* was greener than members of the *M. acridum* clade and did not approach the olive pigmentation of the other members of the complex.

Conidia are the only morphological feature that

reliably distinguishes several *Metarhizium* species considered in this study. The conidia of five of nine species treated here are closely overlapping in size and shape with those of *M. anisopliae* (5.0–7.0 × 2.0–3.5 µm, FIG. 8) and with each other, including *M. brunneum* (4.5–8.0 × 2.0–3.0 µm, FIG. 12), *M. lepidiotae* (5.0–7.0 × 2.0–3.5 µm, FIG. 14), *M. pingshaense* (4.5–5.0 × 2.0–3.5 µm, FIG. 6) and *M. robertsii* (5.0–7.5 × 2.0–3.5 µm, FIG. 10). So it is not possible to distinguish these taxa based on conidial morphology. Isolates in the MGT clade have the largest conidia in the *M. anisopliae* complex, and within the MGT clade *Metarhizium majus* has the largest conidia (FIG. 2), which are cylindrical and 8.5–14.5 × 2.5–5.0 µm. In general the conidia of *M. majus* are rarely less than 10 µm long and are usually 12–13 µm. The second largest conidia of the complex belong to the *M. guizhouense* (= *M. taii*) clade. Conidia of this group are 6.5–10.0 × 2.0–3.5 µm (FIG. 4), but rarely exceed 9 µm long. *Metarhizium acridum* has the smallest conidia of the complex (FIG. 16). They are generally ovoid and are 4.0–5.5 × 2.0–3.0 µm. However the conidial measurements of *M. acridum* isolate ARSEF 324 differed from its conspecifics. The conidia of ARSEF 324 were similar in size to *M. lepidiotae* isolates, although often broader, 5.0–7.5 × 3.0–4.5 µm. In addition the globose conidia of *M. globosum*, which are 4.0–5.5 µm diam, are a distinguishing characteristic of this species. They do not resemble the conidia from any other species in the *M. anisopliae* complex or the genus as a whole.

There was a great deal of variation in the size of phialides within all recognized species. However there did however appear to be a slight positive correlation between phialidic and conidial sizes, although this association is too weak to be taxonomically diagnostic.

DISCUSSION

The species of *Metarhizium*, as in many hyphomycetous genera, can be difficult to distinguish morphologically (Crous et al 2005, Rehner and Buckley 2005, Tsui et al 2006). It has been argued recently that morphological species recognition (MSR) regularly under diagnoses evolutionarily meaningful species (Taylor et al 2000). Thus morphological similarity between sister and closely related species might be the result of faster rates of reproductive isolation and genetic divergence relative to the rate of morphological change (Taylor et al 2006). However, if rates of morphological evolution are heterogeneous among recently diverging species lineages, morphological crypsis among non-sister lineages also could be due to retention of symplesiomorphic morphologies. On the other hand phenotypic similarities among non-sister species might result from convergent morphological evolution (Crous et al 2005, Rehner and Buckley 2005, Tsui et al 2006), perhaps due to occupation of similar ecological niches. In the case of the *M. anisopliae* complex one or more of these phenomena might occur because among the species considered here morphological crypsis occurs between both sister and non-sister taxa.

Our approach to dealing with morphological crypsis in *M. anisopliae* is to explore the use of alternative methods of species recognition. Application of the biological species recognition criterion (BSR) to determine species boundaries as defined by the ability to interbreed unfortunately is currently not an option with *Metarhizium*. While readily cultivated in its mitotic state, neither sexual crosses nor sexual development have been successfully induced in vitro. Moreover BSR has been criticized because the ability to mate represents a single, symplesiomorphic characteristic (Rosen 1979) that can be critically evaluated only in the context of a robust phylogenetic hypothesis. By contrast the genealogical concordance phylogenetic species recognition criterion (GC-PSR, Taylor et al 2000) by itself has proven extremely effective at recognizing diversity within the *M. anisopliae* complex in the absence of diagnostic morphological characters and the present inability to perform in vitro mating tests. GC-PSR has been used regularly to delimit closely related and morphologically undifferentiated species in a wide range of fungi (Koufopanou et al 1997, Geiser et al 1998, O'Donnell et al 1998, 2004, Adam et al 1999, Chaverri et al 2003, Dettman et al 2003, Weber et al 2003, Miller and Huhndorf 2004). Altogether our analyses provide support for the recognition of nine distinct phylogenetic species. As in several of the studies cited above not all gene partitions that we analyzed support the delimitation of each *Metarhizium* species recognized herein. However a minimum threshold of two-gene support was achieved for the recognition of seven phylogenetic species. Where a two-gene minimum support threshold was not obtained (e.g. delimitation of *M. majus* from *M. guizhouense*) fixed differences in conidia morphology and a single gene phylogeny were used as the basis for their recognition. We note that congruence of only one sequenced gene and either phenotype or geographic endemism is commonly invoked as the basis to propose species status in fungi (James et al 2001, Roets et al 2007, Zhao et al 2008).

The core of the *M. anisopliae* complex is represented by the PARB clade. The clade contains the type species of the genus, *M. anisopliae*, and consists of four well supported species lineages that we have recognized here as *M. pingshaense*, *M. anisopliae*, *M.*

robertsii sp. nov. and *M. brunneum* (FIGS. 21–22). All four species are global in distribution, and the isolates examined in this study originated from Asia, Australia, Europe and North and South America. Because few isolates from Africa were available at the time of this study our knowledge of *Metarhizium* from this continent remains fragmentary. With the ability to separate these cryptic species using objective phylogenetic criteria it is now possible to mount systematic efforts to search for additional physiological and ecological features that might further differentiate these phylogenetic species. The genealogical data presented here does not clarify the historical phylogeography of any of these species, which is obscured by their ubiquitous distributions. Determining the centers of origin and dispersal histories of these species will require further analysis requiring both phylogenetic and population genetic approaches.

Metarhizium brunneum is the most basal lineage in the PARB clade. As with other members of the clade we found it impossible to differentiate isolates of *M. brunneum* from *M. anisopliae*, based on morphological characteristics, with the exception of the presumptive color mutant ARSEF 2107. Petch (1935) designated a type collection from the Philippines, which he described as turning brown in mature colonies. This color variant may occur regularly in nature based on the fact that Petch had identified multiple isolates as *M. brunneum* and from geographically distant locations (see collections BPI 447601 and BPI 447602). However because the type specimen of the species is not easily available for examination and because no ex-type culture exists we have designated ARSEF 2107 (Oregon, USA), an isolate that was authenticated by Petch, as the basis for an epitype. It is important to note that the majority of *M. brunneum* isolates examined here possess the typical olivaceous *M. anisopliae* color instead of the buff or tan pigmentation as described for the type specimen or the ex-epitype culture, respectively.

The distinction between *M. majus* and *M. guizhouense* is difficult to interpret because their relationship was not conclusively resolved with the molecular data at hand. Based on morphological observations and especially the results from the 5′ end of EF-1α (FIG. 22), we conclude that there is sufficient evidence to segregate the associated isolates into the two species. From the standpoint of morphology the conidia of *M. guizhouense* were consistently smaller than those of *M. majus*. No isolate of *M. guizhouense* produced conidia larger than 10 μm while all isolates of *M. majus* produced conidia greater than 10 μm long. Thus the morphological data support the recognition of *M. majus* and *M. guizhouense*. However the conflicting topologies between the RPB2 and Bt gene trees (not shown) contradict the monophyly of these taxa. As mentioned above we recognize that acceptance of *M. majus* and *M. guizhouense* at the species rank does not meet the molecular genealogical concordance criteria that we have implemented in recognizing the other taxa within the *M. anisopliae* complex. However the congruence of the morphological grouping based on conidial size with the 5′ EF-1α phylogeny provides a sufficient basis with which to distinguish and to accept these two species. It is possible that the incongruity seen between the RPB2 and EF-1α phylogenies might reflect incomplete sorting of ancestral polymorphisms within this lineage. More detailed phylogenetic study of this clade incorporating additional phylogenetic markers and increased taxon sampling is needed to further evaluate the status of these two species.

We determined that *M. taii* is a synonym of the earlier published name *M. guizhouense* (MGT clade) and suggest that *M. pingshaense* (PARB clade) should be resurrected. Huang et al (2005a, b) presented the first molecular phylogenetic studies to include *M. guizhouense*, *M. pingshaense* and *M. taii* in a context that included additional *Metarhizium* species. With ITS sequence data in both papers they reported a taxonomically broad polytomy for what we define as the *M. anisopliae* complex and concluded that *M. guizhouense*, *M. pingshaense* and *M. taii* all were synonyms of *M. anisopliae*. They also placed *M. majus* within a broadened concept of the *M. anisopliae* clade but did not include it as part of the synonymy. The superior resolution of the multilocus phylogenetic hypothesis inferred in this study has enabled further insight into the phylogenetic and taxonomic status of these species. For example in the present study isolates previously identified as *M. taii* (ARSEF 5714 and ARSEF 6238) were consistently placed in the same clade as the ex-type of *M. guizhouense*. To confirm that our isolates were truly *M. taii* we evaluated their morphology in comparison to other accounts of the taxon and found that the conidial measurements of our isolates were in accordance with the measurements described in the protolog for the species (Liang et al 1991, Huang et al 2005a). Furthermore the *M. taii* conidial measurements were consistent with those of the ex-type isolate of *M. guizhouense*. The holotypes of *M. taii* and *M. guizhouense* both were collected in the subtropical Guizhou Province of China, and both were from lepidopteran hosts. Together the morphological, molecular, host and geographic data lead us to conclude that *M. taii* is a synonym of *M. guizhouense* and therefore the anamorph of *Metacordyceps taii* would be *M. guizhouense*, which has nomenclatural priority. Huang et al (2005b) synonymized *M.*

pingshaense with *M. anisopliae* because of their similar ITS sequences and morphologies. However we resurrect *M. pingshaense* (ex-type CBS 257.90) on the basis of the phylogenetic support for this species lineage. Also the conidial measurements of CBS 257.90, while still within much of the range of *M. anisopliae*, regularly surpassed the limits observed among its conspecifics.

Metarhizium lepidiotae is the most basal member of the *M. anisopliae* complex that is also morphologically indistinguishable from *M. anisopliae*. However the molecular data unequivocally support it as the sister lineage of the PARB plus MGT clades. The similar conidial features of *M. lepidiotae* and *M. anisopliae* might represent a symplesiomorphy that diverged in a common ancestor of the MGT clade, whose species produce larger conidia. On the other hand the similarity might have arisen through convergence. Of note, based on our limited sampling, *M. lepidiotae* appears to have the most restricted geographic range of all members in the complex. Of the six *M. lepidiotae* isolates included in this study two were from Papua New Guinea, two from Queensland, Australia, and two from the Australian island of Tasmania.

In our sampling of *Metarhizium acridum* a number of isolates, including ARSEF 7486, are closely related. This species is used regularly as a biological control agent in Africa against grasshoppers and locusts under the trade name Green Muscle® and more recently as Green Guard®. The ex-type of the species, ARSEF 7486 (originally collected from Niger), is the active ingredient of Green Muscle®, while Green Guard® is a formulated product based on an Australian isolate (Milner 2002, CSIRO FI 48 = ARSEF 324). Widespread release of Green Muscle® (i.e. ARSEF 7486) in many parts of Africa might lead one to think that isolates in this clade (FIG. 16) could be clones separated by multiple generations. However four of the nine isolates (ARSEF 3391, 6600, 324 and 5736), for which 5′ EF-1α sequences were determined, are a different sequence haplotype than ARSEF 7486 and thus are unlikely to be its clonal descendants. In addition isolates (ARSEF 6421, 6592, 6597) that do share the same 5′ EF-1α haplotype as ARSEF 5748 either were collected before the release of Green Muscle® or were collected distant from the sites of localized applications in the Sahel and southern and western Africa (www.lubilosa.org). Although *M. acridum* is primarily known from Africa and Australia ARSEF 5748 was collected in Mexico in 1992 before the widespread use of Green Muscle® (Lomer et al 2001). This suggests that *M. acridum* is more geographically widespread and might occur naturally in environments where locusts and grasshoppers are endemic.

The newly described species *Metarhizium globosum* is distinguished from its sister taxon, *M. acridum*, based on the molecular, host affiliation and morphological data. Both the multigene and 5′ EF-1α phylogenetic analyses significantly support its distinction (FIGS. 21–22). *Metarhizium globosum* is the only taxon within the genus to produce globose conidia. Furthermore *M. acridum* has been collected only from orthopterans and soil isolations while *M. globosum* was isolated from a lepidopteran host. Despite having only a single isolate of *M. globosum* the molecular and morphological data support its distinction as a new species and hopefully will stimulate efforts on the Indian subcontinent to search for additional isolates of this species.

Bidochka et al (2001, 2005) used sequence-based analyses to propose the existence of cryptic species within *M. anisopliae* s.l. from eastern Canada. In addition the usefulness of the nuclear ribosomal intergenic spacer (IGS) region as a phylogenetic marker was evaluated by Pantou et al (2003) and used to characterize the *Metarhizium* diversity associated with Neotropical leaf-cutter ant nests (Hughes et al 2004). Cross-referencing the results of these investigations to each other and to the present study is difficult due to the different genes and isolates sampled and to the lack of reference sequence data for taxonomically validated isolates. We have addressed this issue in two ways to determine the identities of species in these studies. In the case of the Bidochka et al (2001, 2005) we sequenced isolates obtained from the authors and determined that the two mutually exclusive groups they reported are *M. robertsii* and *M. brunneum*. Isolates from Pantou et al (2003) were not readily available, thus we determined IGS sequences for the isolates of the six species in the PARB and MGT clades of this study and compared these to GenBank records submitted by Pantou et al (2003) and referenced by Hughes et al (2004). BLAST (Altschul et al 1990) analyses and an alignment combining our sequences with those of Pantou et al (2003) revealed this correspondence among these studies: (i) Group A of Pantou et al corresponds to our PARB clade, however the IGS of *M. pingshaense* and *M. robertsii* are not reciprocally monophyletic, and thus IGS is not informative for differentiating species within this clade; (ii) Group B of Pantou et al is *M. brunneum*; and (iii) Group C corresponds to the MGT clade, however IGS does not support the monophyly of either *M. guizhouense* or *M. majus*, as with many of the loci sequenced in this study. This comparison demonstrates the potential and need for adoption of standardized molecular identification methods to expedite communication concerning these organisms.

The multigene phylogenetic approach taken in this study of the *Metarhizium anisopliae* complex has shed considerable light on this enigmatic group of fungi. The morphology of species in the complex provides limited diagnostic information for the lineages resolved in the molecular analyses. This is especially true with colony pigmentation. Other than the extended period of time in which *M. acridum* produces yellow pigmentation in culture, the vast majority of isolates of all species showed various shades of olive at maturity. Rombach et al (1986) suggested that phialide morphology might be useful in discerning the taxa of *Metarhizium*, but like Glare et al (1996) we found phialide morphology to be plastic within lineages and not particularly useful for taxonomic diagnoses. We agree with Glare et al (1996) and Driver et al (2000) that conidial morphology, although useful in some cases (i.e. *M. globosum*) is also of limited use for species delimitation and identification. *Metarhizium majus* had the largest conidia followed by *M. guizhouense*, whereas *M. acridum* generally had the smallest (except for ARSEF 324). However distinguishing *M. anisopliae*, *M. pingshaense*, *M. robertsii*, *M. brunneum* and *M. lepidiotae* based on morphology alone was not possible in our examinations of these taxa.

We have found that molecular tools and analyses are the most reliable way to differentiate species within the *Metarhizium anisopliae* complex. Based on the results of this and a study of *M. frigidum* by Bischoff et al (2006), the 5′ region of EF-1α is to date the most informative region to use for routine species identification within the genus. This region requires only two primers and is easily amplified. Although EF-1α alone is not sufficient for fully resolving the genealogy of *Metarhizium*, it is currently the most useful single locus for diagnosing terminal groups as Geiser et al (2004) found for identifying *Fusarium* species. Future studies will determine the use of this single locus for the recognition and identification of phylogenetic species in other fungal species complexes.

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